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SV40 vectors carrying minimal sequence of viral origin with exchangeable capsids

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ABSTRACT

Polyomaviral vectors are generated by transfecting 293T cells with three sets of DNAs: DNA for the expression of simian virus 40 (SV40) T antigen; DNA for the expression of SV40 capsid proteins, and vector DNA harboring a reporter gene expression cassette carrying a SV40 origin. The vector DNA harbors a minimal sequence originating from SV40, and thus can carry a longer transgene. Moreover, the viable recombinants are not detectable in the vector preparation, and the vectors can transduce the DNA with efficiency similar to that of virions. Vector particles bearing capsid proteins of BK virus, JC virus, and B-lymphotropic papovavirus instead of SV40 were prepared, and they exhibited differential efficiency of gene transduction to the target cells. This method can be used to develop a surrogate system to study the functions of capsid proteins of polyomaviruses and to generate a set of polyomaviral vectors targeted at specific cell types.

Introduction

Simian virus 40 (SV40) is a polyomavirus, a non-enveloped small DNA virus whose capsid is composed of major capsid protein Vp1 that forms a pentamer, 72 of which are arranged as an iosahedral capsid shell, and minor capsid proteins Vp2 and Vp3, one of which resides in the inner cavity of the Vp1 pentamer (Imperiale and Major, 2007). Packaged inside the capsid in a minichromosomal form is the double-stranded circular viral DNA of about 5 kbp in size, which can be divided into three elements: (1) the early region, encoding T antigen proteins essential for viral DNA replication and viral gene expression; (2) the late region, encoding Vp1, Vp2, Vp3, and agno protein; and (3) the regulatory region, including the origin of replication and promoters for the expression of genes in the early and late regions. In addition to representative polyomaviruses, such as SV40, murine polyomavirus, JC virus (JCV), BK virus (BKV), and monkey B-lymphotropic papovavirus (LPV), three new human polyomaviruses have been recently reported: Merkel cell polyomavirus (Feng et al., 2008), associated with the rare skin cancer Merkel cell carcinoma and two related viruses, WU virus (Gaynor et al., 2007) and KI virus (Allander et al., 2007), which were described in clinical specimens.

The built-in affinity of viral structural proteins for the natural target cells is often the determinant of virus tropism. Among the polyomaviruses, JCV and LPV exhibit highly restricted cell tropism. JCV infection is restricted to oligodendrocytes, astrocytes, and B-lymphocytes (Assouline and Major, 1991; Major et al., 1990; Major et al., 1985; Major and Vacante, 1989), whereas LPV only infects cells of the B-lymphocyte lineage (Brade et al., 1981; Takemoto et al., 1982). Restriction involves the ability of the viral capsid protein to interact with cell surface receptors (JCV, (Chen and Atwood, 2002; Wei, Liu, and Atwood, 2000); LPV, (Haun et al., 1993; Herrmann, Oppenlander, and Pawlita, 1995)). Thus, one would expect that gene transfer performed using such capsids to be restricted to group of cells of the natural target.

SV40 has been studied as a viral vector with an emphasis on the ease of producing high-titer stocks (Strayer and Milano, 1996) and its ability to transduce genes to many cell types (Kimchi-Sarfaty et al., 2002; Strayer et al., 2002). The vector can package up to 7 kbp of circular DNA, albeit poorly when the size exceeds 5 kbp (Oppenheim et al., 1992), and partial degradation of the oversized DNA upon packaging is also observed (Chang and Wilson, 1986). However, transgene size that can be carried into the vector is only a few kilobases (Strayer, 1996), owing to the built of the vector DNA that was developed from a defective viral variant lacking the T antigen coding sequence (Oppenheim and Peleg, 1989). The vector efficiently propagates in Cos cells constitutively expressing T antigen from defective SV40 DNA integrated into the cellular DNA. It is also prone to the generation of wild-type like recombinants, likely because of recombinations between the vector DNA and defective viral DNA in the host DNA. Such deficiency encouraged the production of alternate cell lines constitutively expressing T antigen driven by non-SV40

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promoters, thus minimizing the risk of recombination (Arad et al., 2002). Alternatively, in vitro encapsulation of DNA using recombinant SV40 capsid proteins was used to generate vector particles (Kimchi-Sarfaty et al., 2002).

Here we made polyomaviral vectors that were able to carry longer transgenes without detectable contamination with wild-type-like recombinants. Using this method, we were able to use capsids of any one of the polyomaviruses SV40, JCV, LPV, and BKV to package the vector DNA. Such vector particles exhibited distinct and differential abilities to transduce DNA to the target cells depending on the capsid used. The method can be used for generating both surrogate systems for studying viral capsid proteins of other polyomaviruses and cell type-restricted gene transfer vectors.

Results

Vector particle production by transient transfection to 293T cells

First, SV40 vectors were made by a transient transfection-based approach. Four DNA constructs, pCI-Ts, pTRE-Vps, mpTet-on, and pSEAP2, were used to generate SV40 vectors. pCI-Ts is used to express SV40 T antigen, which promotes the replication of DNA carrying the SV40 origin and enhancer elements (SV40 Ori), the sequence of which also serves as a packaging signal (Oppenheim et al., 1992). pTRE-Vps, harboring SV40 capsid protein genes under the control of the tetracycline-inducible promoter, and pmTet-on, which expresses the tetracycline-dependent transcriptional activator (rtTA), were introduced together for the expression of capsid proteins upon addition of the tetracycline analogue 24 h post-transfection (hpt). pSEAP2 carries SV40 Ori that also serves as the promoter for expressing the secreted form of embryonic alkaline phosphatase (SEAP). The four DNAs were transfected into 293T cells to generate the vectors. Similar sets of DNAs in which pSEAP2 was replaced with pUC19 were transfected into the cells to produce "empty" particles that did not package vector DNA. SV40 viral DNA was independently transfected into 293T cells to produce wild-type virion. The transfected cells were collected at 68–72 hpt and were extracted for particles. The extracts were then sedimented through a 27%–39% Optiprep continuous gradient. Eighteen fractions were taken from the top of the gradient, and each fraction was examined for the presence of Vp1 by Western blotting (Fig. 1A). Upon sedimenting the extract of cells transfected with SV40 DNA, a Vp1 peak appeared near the bottom of fractions 13–16, indicating the production of fast sedimenting species, most likely virion, in the cells (Fig. 1A, top panel, SV40). Similarly, sedimented fractions of the vector particle preparation showed a Vp1 peak in fractions 13–17 (middle panel, pSEAP2). The fractions of the empty particle preparation showed peaks in fractions 13–16 (bottom panel, No vector DNA), though the Vp1 peak was slightly shifted toward the top, judged from the intensity of the Vp1 bands.

Vector particles in the Vp1 peak fractions were detected for their ability to transduce DNA. An aliquot of each fraction was applied to 293FT cells, and the transduction of DNA was estimated by the expression of SEAP secreted into the culture medium. High alkaline phosphatase activity was found in the culture medium of cells exposed to Vp1 peak fractions 14–17, whereas for cells exposed to fractions 7–13, there was marginal activity in the medium (Fig. 2B). These results imply that the Vp1 peak fractions contain vector particles that can transduce pSEAP2 DNA.

We estimated the ratio of capsid protein to vector DNA in the Vp1 peak fractions to see if the composition of the vector particles was similar to that of the virions. The respective peak fractions were pooled and the amount of vector DNA was estimated by quantitative PCR. As a control, particles of SV40-luc, a SV40 vector of the first generation, were prepared by co-transfecting 293T cells with pCI-Ts and SV40-luc DNA. The particles were purified by Optiprep sedimentation and the Vp1 peak fractions were pooled. The amount of DNA containing SV40 origin sequence in the pooled Vp1 peak fractions was 1.8×10^{11} copies/ml for virions, 1×10^{9} copies/ml for pSEAP2 vectors, and 4.2×10^{9} copies/ml for SV40-luc vectors, but not surprisingly, such DNA was undetectable in fractions with empty particles. Based on the DNA copy number each aliquot of Vp1 peak preparation was adjusted to contain a similar amount of DNA and then examined for the presence of Vp1 and Vp3 by Western blotting. The amounts of Vp1 and Vp3 found in the Vp1 peak preparations were similar among virions (Fig. 1C; SV40; lanes 1, 2, 3, and 10), vector particles (pSEAP2; lanes 4, 5, 6, and 11), and SV40-luc (SV40-luc; lanes 7, 8, 9, and 12). We also tried to examine the amount of histones, the virion component complexed with viral DNA, in the pooled peak fractions by Western blotting; though this was unsuccessful because of low selectivity and sensitivity of the available antibodies to the antigens (data not shown). However, examination of pooled peak fractions by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by

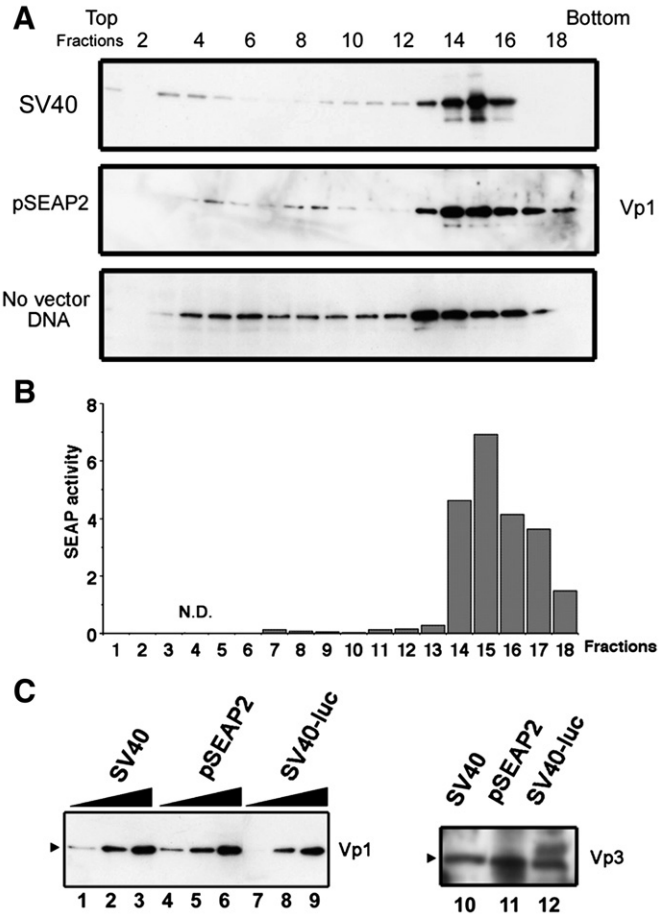

Fig. 1. Production of particles packaging vector DNA. (A) Lysates of the 293T cells transfected with DNAs for producing virion (SV40, top panel), vector particles packaging pSEAP2 DNA (pSEAP2, middle panel), and empty particles (No vector DNA, bottom panel) were sedimented through Optiprep gradients and fractionated from the top. A 10-μl aliquot of each fraction was detected for SV40 Vp1 by Western blotting. (B) Detection of DNA transduction activity. Ten microliter aliquots of each fraction from pSEAP2 in (A) were applied to 293FT cells, incubated for 48 h, and SEAP activity in the 15-μl cell culture medium was measured. Vertical axis represents arbitrary values of relative SEAP activity in the medium. (C) Composition of capsid proteins in the Vp1 peak fractions. The Vp1 peak fractions of SV40 (SV40, lanes 1, 2, 3, and 10), vector particles (pSEAP2, lanes 4, 5, 6, and 11), and SV40-luc (SV40-luc, lanes 7, 8, 9, and 12) were adjusted for DNA, separated by SDS-PAGE, and examined for either Vp1 (left panel) or Vp3 (right panel) by Western blotting. Samples containing 1×10^{5} (lanes 1, 4, and 7), 3×10^{5} (lanes 2, 5, and 8), and 1×10^{6} (lanes 3, 6, and 9) copies of the DNA were applied to each lane. In the right panel (lanes 10–13), samples contained 5×10^{7} copies of the DNA for virion (SV40, lane 10), vector particles (pSEAP2, lane 11), and SV40-luc (SV40-luc, lane 12).

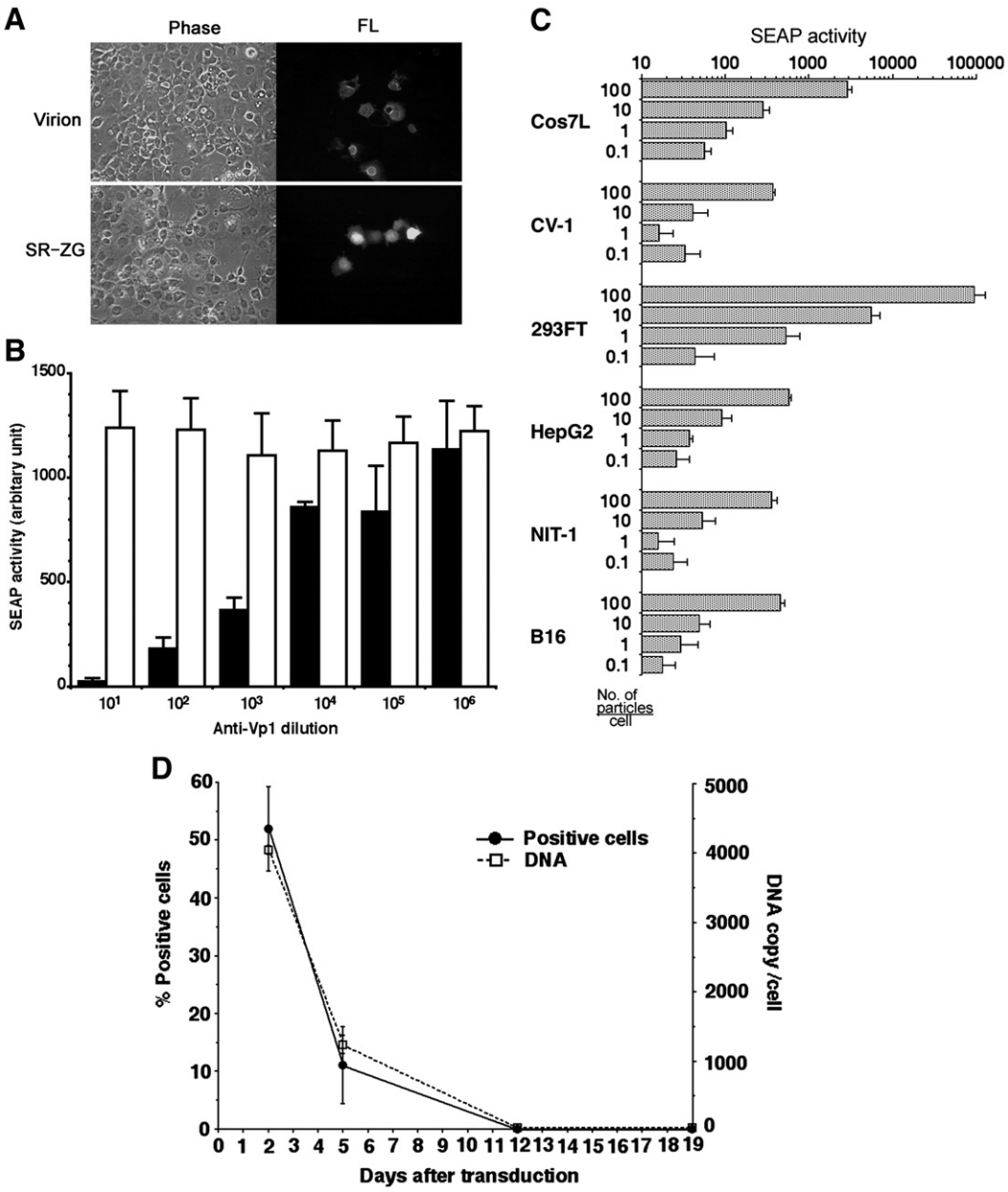

Fig. 2. DNA transduction by vector particles. (A) Transduction efficiency of SV40 virion and SV40 vector particles carrying pSR-ZG (SR-ZG) was examined by challenging Cos7L cells. Virion-infected cells were detected for Vp1 by indirect immune fluorescence technique. Identical sets of cells were visualized for either fluorescence (FL) or by phase-contrast microscopy (Phase). (B) 293T cells grown in 96-well plates were challenged with 2×10^3 pSEAP2-carrying vector particles per cell in the presence of serially diluted rabbit anti-Vp1 IgG (filled bars) or rabbit anti-mouse IgG (open bars). The conditioned medium was used for measurement of SEAP activity after 48 h of incubation. Horizontal and vertical axes indicate fold dilutions of anti-Vp1 serum and arbitrary units of SEAP activity, respectively. (C) pSEAP2 DNA transduction by SV40 vector particles. Approximately 2×10^4 293T, CV-1, Cos7L, NIT-1, and B16 cells grown in 96-well plates were applied with 10 μl of serially diluted vector particle preparations containing 2×10^6, 2×10^5, 2×10^4, and 2×10^3 copies of vector DNA, corresponding to 100, 10, 1, and 0.1 particles/cell, respectively, and the conditioned medium taken 48 h after transduction was used for measuring SEAP activity. Four sets of wells were used to infect the same numbers of particles, and the mean value and standard deviation of the SEAP activity in each set of infections are shown. (D) Transgenes delivered by the SV40 vector did not survive in the long term. 293T cells were transduced with the SV40 vector carrying pSR-ZG, and on days 2, 5, 12, and 19, the cells were examined for ZsGreen-positive cells and the amount of vector DNA by PCR. Closed circles represent proportions of cells positive for ZsGreen fluorescence and open squares represent copy numbers of vector DNA per cell examined from the extracted DNA.

Coomassie blue staining showed characteristic bands at similar positions as histones along with Vp1 and Vp3 (Fig. S1, Supplementary data). Together we concluded that we were able to produce SV40 vectors packaging pSEAP2 DNA.

Transduction of vector DNA into cells

To find out whether the vector particles could transduce DNA as efficiently as virion, vector particles carrying pSR-ZG were generated. pSR-ZG harbors the expression cassette of a green fluorescent protein, *Zoanthus* Green (ZsGreen), driven by the SV40 early promoter with the HTLV enhancer element (Takebe et al., 1988). Vector particles carrying pSR-ZG and virions were purified by Optiprep sedimentation, pooled for the Vp1 peak fractions, and dialyzed to remove Optiprep. The number of vector particles or virions estimated by quantitative PCR for DNA was adjusted and used to infect Cos7L cells. When 10^7 vector particles in 1-ml medium were applied to 10^5 cells for 1 h at 37 °C, and then incubated with 5-ml medium for 48 h, about 11.2% of cells became ZsGreen-positive (Fig. 2A, bottom panels). When applied with similar concentration of the virion preparation, about 10.3% of them

became positive for expression of the viral late gene Vp1, as detected by immunocytochemistry (Fig. 2A, top panels). The results suggest that the vector particles were able to transduce DNA with efficiency similar to that of virion.

To confirm that DNA transduction was mediated by SV40 capsid, 293T cells were challenged with SV40 vector particles carrying pSEAP2 in the presence of antibody against either SV40 Vp1 or mouse IgG (Fig. 2B). In the presence of the highest concentration of anti-Vp1, almost no SEAP activity was found upon transduction by vector particles, and as the amount of anti-Vp1 decreased, the SEAP activity was gradually restored (Fig. 2B, filled columns). The SEAP activity was essentially unchanged in the presence of any concentration of an unrelated antibody, anti-mouse IgG (open columns). These results indicate that transduction was mediated by the SV40 Vp1 protein of the vector particles.

The SV40 vector has been shown to transduce DNA to a variety of cell lines (Vera and Fortes, 2004). To determine whether vector particles had a similar ability, vectors carrying pSEAP2 DNA were used to challenge various cell lines: the SV40 permissive monkey cell lines (Cos7L and CV-1), a SV40-semipermissive human cell line (293T), a hepatoma cell line (HepG2), and non-permissive rodent cell lines (melanoma cell line B16 and pancreatic beta cell line NIT-1). As shown in Fig. 2C, SEAP activity detected in the cell culture medium increased as a function of the number of particles used to challenge the cells. The higher activities detected in Cos7L and 293T cells were probably due to the presence of constitutively expressed T antigen proteins that could enhance SEAP expression driven by the SV40 promoter. We did not see such enhancement in NIT-1 cells, which carry T antigen genes under the control of the insulin promoter, possibly relates to inability of SV40 T antigen to promote viral DNA replication in mouse cells. Nevertheless, the SV40 capsid-based vector particles exhibited a broad host range for DNA transduction.

The episomal nature of polyomaviral DNA replication suggests that without functional T antigen, the vector DNA transduced into the cells would not survive long. 293T cells transduced with SV40 vector carrying pSR-ZG were examined for the proportion of transgene-positive cells upon prolonged culture (Fig. 2D). Not surprisingly, the proportion of cells positive for ZsGreen had diminished significantly 5 days after transduction and became undetectable by 12 days. The vector DNA detected in the cells had also diminished 12 days after transduction, indicating that transgenes delivered by the SV40 vector do not survive long.

Use of vector DNA carrying minimal sequence of viral origin can reduce the chance of generating viable recombinants

In our method of generating vectors, the SV40 viral elements are separated into three DNAs. Therefore, at least two recombination events must occur in order to reconstruct the viable wild-type-like genome. We expected that our vector production method would be less prone to the generation of such recombinants. To this end, we prepared pSCMV-ZG carrying minimal SV40 sequence; the only SV40 sequence was 243 bp encompassing SV40 Ori for replication and packaging and the remaining sequence comprised the ZsGreen expression cassette and the bacterial plasmid sequence. Vector particles carrying pSCMV-ZG were generated in a manner similar to that described above, by transfection to 293T cells. To represent first-generation SV40 vector, particles carrying SV40-luc were prepared by transfecting SV40-luc DNA into Cos7L cells and harvested 72 hpt. Both particles were purified and quantified for vector DNA to estimate the particle number, and were applied to CV-1 cells to test their ability to make plaques; the presence of a viable recombinant would be apparent by the generation of plaques. No plaques were visible after challenge with the vector particle preparation in three independent trials, even at 1×10^{10} particles per well plated with about 5×10^{5} cells. The SV40-luc particle preparation contained viable virus; application of about 1×10^{10} particles generated 3.67 ± 2.08 plaques in three trials, though all plaques were smaller (<1 mm in diameter) than those obtained with the wild-type virion (2.73 ± 0.52 mm in diameter). Not surprisingly, when CV-1 cells were infected with SV40 virion that had been prepared in 293T cells, 1×10^{10} virions yielded about 9×10^{6} plaques. Thus, our method can produce vector particles without detectable contamination with wild-type-like recombinants.

Use of viral capsid of other primate polyomaviruses

We examined if the capsid of non-SV40 polyomaviruses could be used to prepare vector particles. In this experiment, the late gene regions of SV40, JCV, LPV, and BKV were cloned into the pCAGGS plasmid, the expression of which was driven by a hybrid of the hCMV enhancer and the chicken beta-actin promoter. Each capsid expression

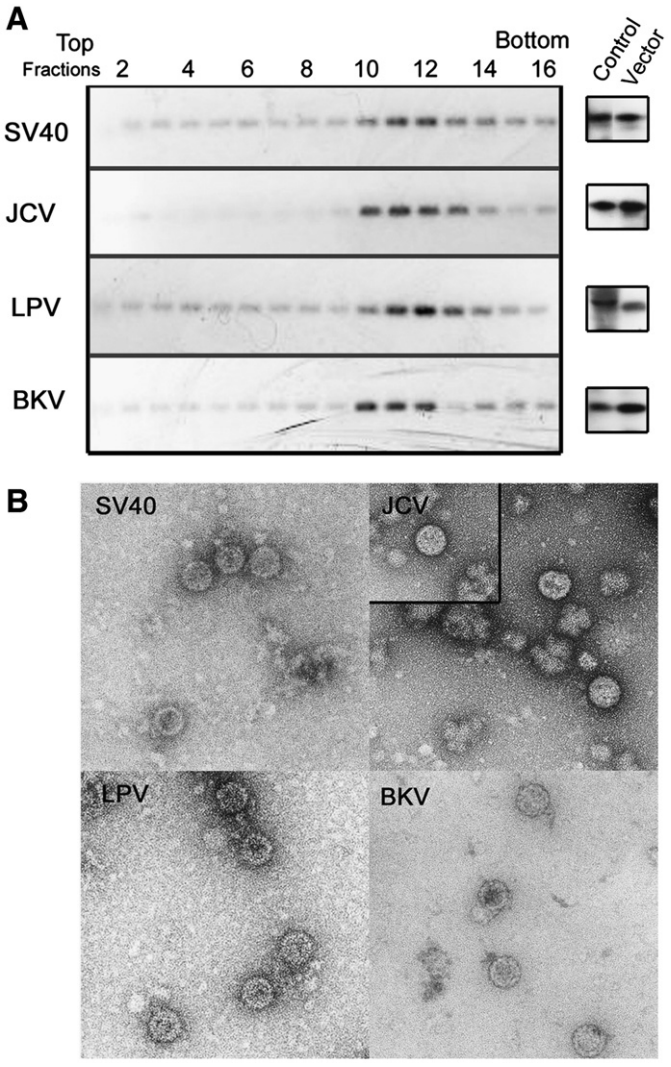

Fig. 3. Use of non-SV40 polyomaviral capsid to produce vector particles. (A) Lysates of 293T cells transfected with pSCMV ZG, pCI-Ts, and pCAG DNA carrying expressing cassette for one of capsid proteins of SV40, JCV, LPV, and BKV were sedimented through 27%–39% Optiprep gradients and fractionated from the top. An aliquot of each fraction was examined for the presence of vector DNA by PCR, amplifying the SV40 Ori region. In the right column, pooled DNA peak fractions of vectors (Vector) and the corresponding infectious particles (Control), adjusted to contain similar amounts of DNA, were examined for the amount of Vp1 using the respective antibodies (see Materials and methods). (B) Vector DNA peak fractions were pooled, pelleted by high-speed centrifugation, resuspended to contain 10^{7}–10^{8} vector DNA copies/μl. The samples were fixed with 2% glutaraldehyde and placed on a glow discharge carbon-coated grid, stained with 0.2% uranyl acetate, and observed with a Joel JEM1200EX electron microscope at 100 kV.

construct was transfected into 293T cells with pCI-Ts and pSCMV-ZG, and the cell extract was sedimented through a 27%–39% Optiprep gradient. Sixteen fractions were taken from the top and each was examined for the presence of vector DNA by PCR. Fig. 3A shows the agarose gel electrophoresis profile of the PCR product amplified for SV40 origin. Presence of DNA was apparent in fractions 10 to 13, and the profile was similar among all vector preparations. The DNA peak of SV40 vectors was also collinear with that of Vp1 detected by Western blotting (data not shown). The DNA peak fractions were pooled, quantified for DNA, and compared with the capsid protein present in the preparations along with the purified virions or infectious particles (see Materials and methods). Both vector and the infectious particles were adjusted to contain similar amounts of DNA and tested for Vp1 by Western blotting (Fig. 3A, right columns). All vector preparations (Vector) appeared to contain similar amounts of Vp1 as the viral counterpart (Control) suggesting that the packaging efficiency of the vector was comparable to that of the infectious particles. The vector DNA peak pool was then dialyzed to remove Optiprep, pelleted by high-speed centrifugation to concentrate the vector particles, and then observed under an electron microscope (Fig. 3B). Structures closely resemble to virion particles were visible in all preparations. Contaminants that did not resemble polyomaviral particles were present in a JCV preparation whose origin was not known (Fig. 3B, lower panel). Together, the results suggest that capsid proteins of JCV, BKV, and LPV can package vector DNA amplified by SV40 T antigen and form particles.

To find out whether such particles can be used as gene transfer vehicles, particles carrying pSCMV-ZG were used to challenge SV40-permissive TC7 cells, JCV-permissive SVG cells, LPV-permissive Namalwa cells, BKV-permissive Vero cells, and mouse myoblastic cells (C2C12). The proportions of transduced cells are shown in Fig. 4. Upon challenge of TC7 cells (TC7) a ZsGreen signal was readily found in cells transduced with the SV40 vector; the remaining vectors were less efficient in generating ZsGreen-positive cells. Challenge of Vero cells also showed that the SV40 vector was the most efficient in transducing vector DNA (Vero). Similar results were obtained using 293T cells (data not shown). In contrast, when SVG human glial cells were challenged with vectors (SVG), almost all cells showed positive ZsGreen fluorescence after JCV vector transduction, whereas the cells that received SV40 and BKV vectors showed less frequent presence of the positive cells and the least in the cells received LPV vector. When Namalwa cells were challenged, none of the vector particles generated significant number of ZsGreen-positive cells (data not shown). Interestingly, when mouse myoblast C2C12 cells (C2C12) were challenged, LPV vector transduction appeared to be the most efficient, followed by the SV40 vector. The remaining vectors did not generate significant numbers of ZsGreen-positive cells. These results indicate that vector particles prepared from JCV, LPV, and BKV capsid proteins exhibit different abilities in transducing DNA to target cells, SV40 capsid being the most effective for TC7 and Vero cells, JCV for SVG cells, and LPV for C2C12 cells.

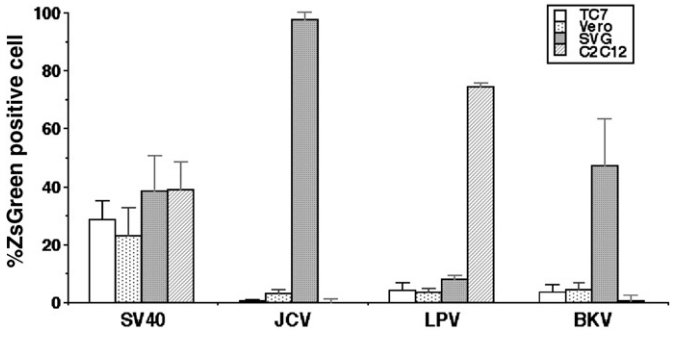

Fig. 4. DNA transduction by vector particles shows different tropism depending on the capsid used. Cells plated on eight-well Labtek chamber slides received approximately 4×10^3 vector particles carrying pSCMV-ZG DNA per cell and were incubated for 48 h before fixation. ZsGreen expression was detected either by the fluorescence of ZsGreen (SVG) or by indirect immunofluorescence (TC7, Vero, and C2C12). The cell nucleus was counterstained with DAPI to show individual cells.

Discussion

We have made SV40-based polyomaviral vectors by transfecting 293T cells with three sets of DNAs: DNA for the expression of SV40 T antigen; DNA for the expression of SV40 capsid proteins, and vector DNA harboring a reporter gene expression cassette carrying SV40 Ori. Vectors generated in the transfected cells were examined by sedimentation through 27%–39% Optiprep gradients and the respective peaks of Vp1 and DNA appeared in similar positions to the virions. When observed under the electron microscope, the peak fractions contained particles morphologically similar to the virion. The composition of SV40 vector particles and the efficiency of transducing DNA to the target cells were comparable to those of the SV40 virion. Our method can produce vectors with no detectable contamination with viable recombinants. Furthermore, with this method we were able to produce vectors bearing capsid proteins of JCV, LPV, or BKV, and the ability to transduce vector DNA to target cells differed among different cell types, reflecting the tropisms of the original viruses. Such polyomaviral vectors with exchangeable capsids would be of benefit in the development of surrogate systems for studying viral capsid proteins of other polyomaviruses and in targeted gene delivery systems.

Our results show that it is possible to prepare vector particles bearing a capsid from any of the four different polyomaviruses—SV40, JCV, BLV, and LPV. Since the polyomaviral capsid is known to play an important role in determining the viral cell tropism (JCV: (Chen and Atwood, 2002); LPV: (Haun et al., 1993; Herrmann, Oppenlander, and Pawlita, 1995), capsid exchange could significantly alter the cell specificity of gene transfer. Indeed, our results indicate that the vectors made of JCV and LPV capsids showed distinct and restricted cell tropisms (Fig. 4). The tropism, however, was not always the same as that of the original virus. In contrast to the tropism revealed in the JCV capsid-bearing vector, which closely resembled to that of the virion, tropism of LPV capsid vector did not strictly follow the tropism of the virions. Unexpectedly, the LPV capsid vector showed efficient transduction into mouse myoblast cells. Although it is not known whether the natural LPV virion can infect myoblasts, C2C12 cells could share common cell surface receptors functionally equivalent to those displayed in LPV-permissive cells. Transduction by BKV capsid-bearing vectors to BKV-permissive Vero cells was moderate and with lesser degree than that of SVG cells. It is known that human fetal glial cells are also permissive to BKV infection (Takemoto et al., 1979), and it is not surprising that SVG cells, originated from human fetal glial cells, readily received BKV vectors. The BKV strain used in our study is from archetypic strain and differences in the amino acid sequences of the capsid proteins with the commonly used strain, MM and Dunlop, are not significant (Nishimoto et al., 2006). It is not known whether such differences influence the cell tropism of the vector.

Other viral vector systems have taken advantage of exchangeable viral capsid components of different serotypes exhibiting different cell tropisms. Adenoviral vectors, most of which are derived from serotype 5, have been modified to carry fiber proteins of serotypes 35 and 11, thus changing the cell surface receptor to which the vector binds from CAR to CD34. This modification allowed the vector to transduce genes to cells that poorly displayed CAR but did display CD34, such as hematopoietic cells (Shayakhmetov et al., 2002; Stecher et al., 2001). Distinct cell tropisms are known among 11 different serotypes of adeno-associated viral vectors, each of which is differentially used depending on the target tissue (Gregorevic et al., 2004; Grimm and Kay, 2003; Nakai et al., 2005). Both vector systems were examined in an in vivo transfer system to determine the degree of targeting to a

specific tissue, the methods of which are yet to be examined in our polyomaviral vectors and will be investigated in future.

We suggest that in 293T cells, the DNA episomally replicated by SV40 T antigen can be readily packaged by non-SV40 capsid proteins. The packaging efficiency of the vector DNA did not differ among different capsids (Fig. 3A). It is known that JCV could do so, since a variant of SV40 whose capsid coding region was replaced with that of JCV forms fully infectious particles (Chen and Atwood, 2002). We show here that capsids of BKV and LPV, i.e, other than those of JCV, were able to package DNA whose replication was mediated by SV40 T antigen. Furthermore, bovine papillomavirus capsid proteins co-expressed with SV40 T antigen can form particles packaging episomally replicated DNA (Buck et al., 2004). Thus, it seems plausible that capsids from other polyomaviruses can be used to generate vector particles. Indeed, the capsids of murine polyomavirus and baboon polyomavirus SA12 can also be used to generate vector particles (data not shown). This suggests that using the method described here, the capsid protein of any polyomavirus could be used to generate vector particles.

The present results show that the efficiency of DNA transduction by SV40 vector particles was similar to that of the virion. A fixed number of particles, judged from the number of DNA copies, was applied to SV40 permissive cells and we found that the proportion of cells transduced with the vector DNA was similar to proportion of cells transduced with the virion. Knowing that the composition of the particles—the molecular ratio of capsid protein to DNA—was similar to that of the virion, it is not surprising that the ability of vector particles to transduce DNA to the cells was equivalent to that of virion. The results indicate that our system can generate vector particles whose competence in transducing the DNA into cells is similar to that of the wild-type virion.

SV40 vectors of the first generation were particularly useful because they are easy to prepare in high-titer stocks (Strayer and Milano, 1996; Vera et al., 2004), but the vector preparations were likely contaminated with viable recombinants (Oppenheim and Peleg, 1989). To prevent such contamination, an adenoviral vector expressing SV40 capsid protein was introduced into Cos7 cells together with a vector DNA carrying SV40 Ori to generate SV40 vectors (Fang et al., 1997). In our system, viral genes were separated into three parts to generate particles, and two recombination events must take place to reconstruct wild-type-like DNA. Our results showed that such events are unlikely to occur in our method. Furthermore, our method allowed the production of the SV40 vector carrying DNA expressing fluorescent proteins, which was not possible previously for unknown reasons (Vera and Fortes, 2004), unless an in vitro packaging system was used (Kimchi-Sarfaty et al., 2003). With minimal SV40-derived sequence, we would be able to design a longer transgene with less complication to that can be carried into vector DNA.

Recently new human polyomaviruses have been discovered: Wu virus (Gaynor et al., 2007) and KI virus (Allander et al., 2007) were found in respiratory secretions and nasopharyngeal aspirate samples, respectively, and Merkel cell polyomavirus was found in a rare skin cancer, Merkel cell carcinoma (Feng et al., 2008). Although it is not known whether these viruses are human pathogens, our vector production method would provide a surrogate system for studying the functions of their capsids in the viral life cycle by which providing a means of examining the roles of these viruses in associated diseases.

Materials and methods

Construction of DNAs

All sequences generated by PCR were verified by sequencing. Relevant restriction sites in the primer sequences described are underlined.

pCI-Ts was constructed by replacing the AvrII-to-ScaI fragment of pSVp23a (Ishii et al., 1994) with the XbaI-to-NruI fragment of pOG44 (Stratagene, La Jolla, CA), harboring the human cytomegalovirus immediate-early (hCMV) promoter to generate T antigen expression plasmid.

All vector DNAs carried SV40 Ori including SV40 origin and enhancer elements spanning SV40 nucleotides 5171–130 (Reddy et al., 1978). pSEAP2, 5115 bp in size, carrying an expression cassette for SEAP driven by the SV40 early promoter, was from Takara-Clontech (Otsu, Shiga, Japan). pSR-ZG, about 4.2 kbp in size, was constructed by inserting an EcoRI-to-NotI fragment from pZs Green-1 (Takara-Clontech) into the corresponding site of pSRalpha (Takebe et al., 1988). pSCMV-ZG, 5509 bp in size, was constructed from pCAG-ZG. Briefly, pCAG-ZG was constructed by inserting the EcoRI-to-Bsu36I fragment from pZsGreen-1, encoding the ZsGreen gene, into the corresponding site of pCAGGS (Niwa et al., 1991). Then, using pCAG-ZG as the template, the fragment harboring the ZsGreen gene and rabbit beta-globin polyadenylation-signal (PolyA) was amplified by PCR using the primers 5′-CAAA GAATTC TGCAGTCGACGGT-3′ and 5′-GATT CTCGAG AGCTTGGGCTGCAGGTCGAGGGAT-3′, digested with EcoRI and XhoI, and ligated with two other fragments—the SpeI-to-EcoRI fragment harboring the hCMV promoter excised from pAltermax II (Promega, Madison, WI, USA) and the KpnI-to-AvrII fragment carrying SV40 Ori from NOpSV40 (Ishii et al., 1994). The triple-ligated fragment was inserted into the KpnI and EcoRI sites of pSEAP2, generating pSCMV-ZG. SV40-luc was constructed by replacing the AvrII-to-BclI fragment encoding T antigen genes of pUCSV40 (Ishizu et al., 2001), with the NheI-to-XbaI fragment of pGL3-basic (Promega) encoding the firefly luciferase gene. In the resulting construct, the AvrII and BclI sites were replaced with the NheI and BglII sites, respectively, by the overlapping mutagenesis technique (Ho et al., 1989). The primers used for this particular cloning can be obtained from the authors upon request. pSV40-luc was digested with BamHI and self-ligated to reconstruct circular vector DNA before use for transfection as described previously (Ishii et al., 1994).

pTRE-Vps was constructed from pTRE-tight (Takara-Clontech) by introducing SV40 late gene region from 294 to 2593 (SV40 nucleotide number) amplified by PCR using NO-pSV40 SRBSM (Li et al., 2003) as the template, and set of primers; the forward primer hybridizing 243–276 and the reverse primer hybridizing 2579–2593 introducing NotI sites at the end of the fragment (5′-TAGTAGGCTA GCGGCCGC AGATGTTCACTGCATTCTAGT-3′). The resulting fragment was digested with KpnI and NotI, and inserted at the corresponding sites of pTRE-tight (Takara-Clontech) to generate pTRE-Vps. pmTet-on was constructed from pTet-on (Takara-Clontech) by XhoI digestion and self-ligation, which removed the fragment harboring SV40 Ori.

pCAG-SV40 was constructed by inserting two fragments via the KpnI–BamHI sites to pCAG-ZG: the KpnI-to-NotI fragment harboring the SV40 late gene region from pTRE-Vps, and the NotI-to-BamHI fragment harboring rabbit globin PolyA from pCAG-ZG (see above); pCAG-ZG had the SV40 origin and PolyA as the second expression cassette, which was removed in the process of constructing pCAG-SV40. pCAG-JCV was constructed from pCAG-SV40 by replacing the KpnI–NotI fragment with a PCR fragment generated using pJC1–4→pJCV (Howley et al., 1980) (Health Science Resources Bank, Osaka, Japan) as the template and the following primers: 5′-ACGTCT GGTACCGGT GGCCATGGTTCTTCGCCAGCTGT-3′ and 5′-ACGTCT GCGGCCGC GGATCCGATTACAGCATTTTTGTCTGCAACTGTCC-3′; the amplifying region carried all late genes of JCV encompassing JCV nucleotides (NCBI accession number J02226) 277–2535. The inserted fragment also carried the AgeI site (5′-ACCGGT-3′) overlapping the KpnI site (5′-GGTACC-3′). Similarly, pCAG-BKV was constructed from pCAG-JCV by replacing the AgeI-to-NotI fragment with the PCR fragment generated by molecular clone of BKV KOM5 (a kind gift from Dr. Y. Yogo, Tokyo University, Tokyo, Japan (Nishimoto et al., 2006); NCBI accession number AB211374) as the template, and primers 5′-AATTAA ACCGGT ATGGTTCTGCGCCAGCTGTCACG-3′ and 5′-TTAATT GCGGCCGC TGTTTAAAGCATTTT GGTTTGCAATTGTCC-3′. pCAG-BKV

harbors BKV agno and the capsid protein genes. pCAG-LPV2 was constructed from pCAG-SV40 by replacing the KpnI-to-NotI fragment encoding the LPV late region, including enhancer elements and the capsid coding region spanning LPV nucleotides 5059–2658 (NCBI accession number M30540), amplification using pLPV-P12 (kind gift from Dr. M. Pawlita, Deutsches Krebsforschungszentrum, Heidelberg, Germany (Haun et al., 1993)) as the template and the primers 5′-NNNNNN ACCGGT GGTACCC TAGGGTTGCCATAGTGATTTTGCAG-3′ and 5′-AATTAA GCGGCCGC TCAGCCTCACATATCATTTGATACAGGGAG-3′.

pSVLPV2 was constructed from NO-pSV40 SRBSM by replacing the late gene region with the LPV capsid coding region from pCAG-LPV2 using the KpnI and NotI sites.

Cells

CV-1, Namalwa, and Vero cells were from the Health Science Research Resources Bank (Osaka, Japan). TC-7 and SVG cells were kindly provided by Dr. H. Kasamatsu, University of California, Los Angeles, CA, USA, and Dr. W. Atwood, Brown University, Providence, RI, USA, respectively. 293T, HepG2, C2C12, NIT-1, and B16-F10 (B16) were from the American Tissue Culture Collection, Washington, DC, USA. 293FT and Cos7L cells were from Invitrogen (Carlsbad, CA, USA). The cells were cultured and maintained according to the instructions provided by the respective sources.

Detection of capsid proteins and DNAs

Capsid proteins were detected by Western blotting as described (Nakanishi et al., 2002). Briefly, the denatured samples were separated by 5%–20% gradient SDS-PAGE, transferred onto PVDF membranes (Immobilon-P; Millipore, Billerica, MA, USA), and probed for the respective rabbit antibodies followed by reaction with HRP-conjugated anti-rabbit IgG antibody (Bio-Rad, CA, USA) and detecting the signal by chemiluminescence (ECL-Plus; GE Healthcare Sciences, UK). Anti-SV40 Vp1 and anti-SV40 Vp3 rabbit antisera, provided by Dr. H. Kasamatsu were used to detect SV40 capsid proteins. Rabbit anti-Vp1 antibodies for JCV and LPV were kindly provided by Dr. Shishido-Hara (Kyorin University, Tokyo, Japan (Shishido-Hara et al., 2004)) and Dr. M. Pawlita, respectively. BKV Vp1 was detected using anti-SV40 Vp1 antiserum through its cross-reactivity with BKV Vp1.

The copy numbers of vector DNA were quantified with a real-time PCR kit (Lightcycler; Roche Applied Biosciences, Basel, Switzerland) using the primer set OriPCR1 (5′-AAGCCTCCTCACTACTTCTGGAATGCTC-3′), which hybridizes the SV40 sequence 5201–5230, and OriPCR2 (5′-AGCATGCATCTCAATTAGTCAGCAACCCATAGTCC-3′), which is complementary to 101–134 amplifying the 174-bp SV40 origin region common to all vector DNAs and SV40 DNA. Hybrid DNA carrying SV40 Ori, SV40 early genes, and capsid protein genes from either JCV or LPV was similarly quantified using the OriPCR primers. BKV DNA was quantified using the primer set 5′-TCTTTCCCCTATTAGGCCCTCAATGG-3′, which hybridizes the BKV sequence 1166–1191, and 5′-CTCTCCTGAATGTA-CAGTTTGTTG-3′, which is complementary to 1325–1302.

Vector particle production and viral particle preparation

Although having integrated copy of the SV40 T antigen expression cassette, 293T and 293FT cells poorly support replication of the transfected DNA carrying SV40 Ori (Buck et al., 2004). 293TT cells (a kind gift from J. Schiller, NIH, Bethesda, MD, USA (Buck et al., 2004)) did support DNA replication, though transient transfection of pCI-Ts gave better results in promoting vector DNA replication in our study (Fig. S2, Supplementary data).

293T cells, plated at 6×10^6 cells in 10 cm dishes, were transfected with one of four sets of DNAs: (A) 4 μg of pTRE-Vps, 2 μg of pmTet-on, 2 μg of pCI-Ts, and 4 μg of either pSEAP2 or pSR ZG; (B), 4 μg of SV40 and 8 μg of pUC119; (C) 4 μg of SV40-luc, 2 μg of pCI-Ts, and 6 μg of pUC119; (D) 6 μg of either pCAG-SV40, pCAG-JCV, pCAG-BKV, or pCAG-LPV2, 2 μg of pCI-Ts, and 4 μg of pSCMV-ZG. The cells were replated onto 15 cm dishes 6–7 h after transfection and incubated for 68–72 h. When transfected with DNA of set A, 5 μg/ml doxycycline was added to induce Vp1 expression 24 h after transfection. After incubation, cells were scraped off with a cell lifter and collected by centrifugation at 700 ×*g* at room temperature for 5 min. The cell pellet was resuspended in 1/40 culture volume of 10 mM Tris–HCl pH 7.5, 2 mM $MgCl_2$, 0.25% Brij 58 (Sigma, St. Louis, MO, USA), 5 U/ml of Benzonase (Sigma), and protease inhibitor cocktail (Nakalai, Kyoto, Japan), and then incubated for 30 min at 37 °C. The lysate was spun down at 2500 rpm at 4 °C for 5 min, and the supernatant was overlaid onto a 27%–39% Optiprep continuous gradient in 10 mM HEPES at pH 7.5, prepared as described by Buck et al. (2004). After ultracentrifugation at 45 krpm, for 3.5 h at 16 °C using a Beckman SW50.1 Ti rotor, 200-μl fractions were taken from the top. To detect vector DNA peak fractions for SV40, JCV, BKV, and LPV capsid vector particles, 1-μl aliquots from each fraction were mixed with 9 μl of 0.02% trypsin and 0.025% EDTA and incubated for 10 min at 37 °C, and 1 μl of the 100-fold-diluted trypsin digest was used as the template to amplify the SV40 origin region of the vector DNA by PCR using PrimeStar enzyme (Takara) and OriPCR1 and OriPCR2 as primers. The reactions were run on 1% agarose gel and the PCR products were visualized by ethidium bromide staining.

The SV40 Vp1 or vector DNA peak fractions were pooled and dialyzed in Dulbecco's PBS (−) to remove Optiprep, and ultracentrifuged using a Beckman SW50.1 Ti rotor at 45 krpm for 1 h at 4 °C. The pellet was resuspended in 10 mM HEPES (pH 7.5) and 10 mM KCl in the presence of protease inhibitor cocktail. The copy number of the vector DNA present in the preparation was quantified by real-time PCR as described above.

SV40 and BKV virions were prepared from the viral DNA generated from the respective molecular clones, pUCSV40 and pUCBKV KOM5 (Nishimoto et al., 2006), by digesting with BamHI and self-ligation. Infectious particles carrying either JCV or LPV capsid were prepared from the corresponding SV40 hybrid because of the difficulty of propagation in cell culture. pBR SVJC (a kind gift from Dr. W. Atwood (Chen and Atwood, 2002)) and pSVLPV2, which carry SV40's Ori and the early genes but encode late genes of JCV and LPV, respectively, were digested with BamHI and self-relegated to generate the respective hybrid viral DNAs. The DNAs were transfected to 293T cells and purified for particles in the same way as for the vectors described above. To obtain enough number of BKV particles 1×10^9 293T cells were transfected with the viral DNA. The DNA peak fractions identified by PCR were pooled and the amounts of DNA were quantified as described (see Detection of capsid proteins and DNAs).

Transduction assay

To measure SEAP activity, 15 μl of conditioned medium was assayed for activity by chemiluminescence-based detection using the SEAP assay kit (Takara) and a plate reader for measuring luminescence (Powerscan HT; Dainippon Sumitomo Pharmaceuticals, Osaka, Japan). To examine reporter or viral gene expression by an immunofluorescence technique, cells grown in eight-well Labtek chamber slides (Nunc, Rochester, NY, USA) were challenged with the respective vectors, fixed with 4% paraformaldehyde in Dulbecco's PBS (−), then reacted with either rabbit anti-ZsGreen antibody (Takara) or rabbit anti-SV40 Vp1 serum followed by reaction with Alexa Fluor 555 goat anti-rabbit antibody (Invitrogen). The cells were observed with an epifluorescence microscope (BX60; Olympus, Tokyo, Japan) to detect either ZsGreen autofluorescence or Alexa Fluor 555 fluorescence.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2008.06.032.

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